The distinctive significance of analgesic drugs and olfactory stimulants on learned pain in mice

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Abstract

Chronic pain is often intractable to analgesics, and in animals it involves a conditioned nociceptive response (CR) – learned pain. The neural pathways of nociception and olfactory function in the brain overlap. The influence of olfactory stimuli on acute pain has been studied in some depth in animal and human models, but the influence of olfactory stimuli on learned pain has not been understood. We examined the effects of analgesic drugs and olfactory stimulants (preferred or repellent odor) on acute pain, the unconditioned nociceptive response (UCR) and the CR in mice. The CR was provoked by repeated injection of formalin into the hind-paw in animals in the same context, which elicited the typical pain behaviors of paw licking (including biting). The analgesic drugs acetaminophen, fentanyl, gabapentin and fluvoxamine diminished the UCR but did not affect the CR. In contrast, the preferred odor reduced both the UCR and the CR. Our findings suggest that, like chronic pain, the CR is resistant to analgesic drugs and that preferred odor suppress the neural pathways that mediate the CR of pain perception.

Keywords: conditioned nociceptive response, learned pain, analgesia, odor olfactory stimulation, mouse

Abbreviations: CR, conditioned nociceptive response; UCR, unconditioned nociceptive response; i.p., intraperitoneal injection; s.c., subcutaneous injection; SSRI, selective serotonin reuptake inhibitor

1. Introduction

Pain is a common and debilitating consequence of many different diseases and disorders, and the management of chronic pain is particularly challenging. The therapeutic effect of many analgesic drugs is disappointing when treating intractable pain; consequently, a multidisciplinary approach is needed to address the substantial impairment in the quality of life of those affected (Dewilde et al., 2009; Vranken, 2009). We have previously shown that intractable chronic pain results in a conditioned, learned response to nociception (Nakama-Kitamura and Kitamura, 2011). Briefly, subcutaneous administration of formalin, an unconditioned stimulus, produces licking (including biting) behavior at the site of injection (the unconditioned response, UCR). Repeated injection of formalin under the same context (conditioned stimulus; tone and box) leads to the development of a conditioned nociceptive response (CR) when saline is injected. The CR persists to the same extent 3 weeks later, but only when the animals are placed in the same context in which they have been conditioned.

This study was designed to examine the effects of analgesic drugs and olfactory stimulants on the CR in mice. The analgesic drugs acetaminophen, fentanyl, gabapentin, and antinociceptive analgesics such as fluvoxamine are all used routinely in the treatment of nociceptive and neuropathic pain, and were administered to animals that had acquired a CR. These drugs are used to treat acute and chronic pain in clinical practice, but are less effective in treating sustained pain in experimental and clinical situations (Clarke et al., 2013; Kilic et al., 2012; Gustavsson et al., 2013).

The neural pathways of acute and chronic pain have been extensively studied (Low and Fitzgerald, 2012; Todd, 2010; Zhuo, 2007; Zhuo, 2013): the spinothalamic tract is the main pathway that transmits nociceptive information from the spinal cord to

supraspinal targets (Gauriau and Bernard, 2004; Zhuo, 2007). In patients with spinal cord injury who experience neuropathic pain, cognitive tasks can activate neural pathways involved in pain processing independently of peripheral inputs (Gustin et al., 2010). Nociceptive information from the lateral spinothalamic tract enters the ventrobasal complex of the thalamus and projects to the prefrontal, orbitofrontal and dorsoparietal cortices through the primary and secondary somatosensory cortices and the insula. The anterior spinothalamic tract reaches the intralaminar complex of the thalamus through the brainstem reticular formation and diffuses through the limbic system, which includes the hippocampus, amygdala and the anterior cingulate cortex (Gabbott et al., 2006; Gauriau and Bernard, 2004; Kim et al, 2010; Neugebauer et al., 2009; Zhuo, 2007).

The use of olfactory stimuli to treat acute pain has been examined in animals and humans (Martin, 2006; Renner and Schreiber, 2012; Stankewitz, 2011). The pain and olfactory pathways, and the reciprocal interactions between them, are similar in rodents and humans (Furuono et al., 2009; Heimann et al., 2013; Nanji et al., 2013). In humans, the magnitude of nociceptive withdrawal reflexes to electrical stimulation of the sural nerve, a defensive response mediated by acute spinal nociception, can be modulated by olfactory stimuli (Bartolo et al., 2013). These studies suggest that the olfactory system may play a role in controlling pain. However, there have been few studies on the effect of olfactory stimuli on sustained or learned responses to pain. It was recently reported that the loss of function of the sodium channel gene encoding the voltage-gated sodium channel Na(v)1.7 (*SCN9A*) in olfactory neurons caused a congenital inability to experience pain in humans (Ahn et al., 2011; Weiss et al., 2011). Na(v)1.7 is not only

necessary for pain sensation, but also for odor perception in mice, rats and humans (Zufall, et al., 2012).

The main purpose of this study was to investigate whether odor affects the CR. Using mice as a model, we compared the effect of antinociceptive drugs and odors on the UCR and the CR.

2. Results

2.1. Experiment 1: The effect of analgesic drugs on the UCR and CR

2.1.1. The effect of acetaminophen on the UCR and CR

On day 1 of the training phase (Fig. 1), intraperitoneal injections (i.p.) of acetaminophen (ac) at doses of 100 and 150 mg/kg reduced the mean time spent licking (including biting) the hind-paw (Fig. 2A). For the UCR, there were significant differences across the groups (four-group ANOVA, $F(3, 28) = 84.1$, $p < 0.001$), with *post hoc* testing revealing significant differences between the control and experimental groups, and between the 100 and 150 mg/kg i.p. acetaminophen groups (Ryan's multiple pairwise comparison test). In the test phase, acetaminophen did not influence the CR (Fig. 2B).

2.1.2. The effect of fentanyl on the UCR and CR

On day 1 in the training phase, 0.01 and 0.025 mg/kg i.p. fentanyl significantly reduced the mean time spent licking the hind-paw (Fig. 3A). For the UCR, there were significant differences across the groups (four-group ANOVA, $F(3, 28) = 25.2$, $p < 0.005$), with *post hoc* testing revealing significant differences between the control and experimental groups, between the formalin and fentanyl groups, and between the 0.1 and 0.25 mg/kg i.p. fentanyl groups. In the test phase, fentanyl did not influence the CR (Fig. 3B).

2.1.3. The effect of gabapentin on the UCR and CR

Gabapentin is used routinely to treat neuropathic pain. On day 1 of the training phase, 5 and 10 mg/kg i.p. gabapentin significantly reduced the mean time spent licking the hind-paw (Fig. 4A). For the UCR, there were significant differences across the groups (four-group ANOVA, $F(3, 28) = 90.9$, $p < 0.005$), with *post hoc* testing revealing significant differences between the control and experimental groups, and between the 5

and 10 mg/kg i.p. gabapentin groups. In the test phase, gabapentin did not influence the CR (Fig. 4B).

2.1.4. The effect of fluvoxamine on the UCR and CR

Fluvoxamine is a selective serotonin reuptake inhibitor (SSRI) and has been used as a hypoalgesic drug. On day 1 of the training phase, 5 and 10 mg/kg i.p. of fluvoxamine significantly reduced the mean time spent licking the hind-paw in a dose-dependent manner (Fig. 5A). For the UCR, there were significant differences across the groups (four-group ANOVA, $F(3, 28) = 29.7, p < 0.005$), with *post hoc* testing revealing significant differences between the control and experimental groups, between the formalin and 5 mg/kg i.p. fluvoxamine groups, and between the 5 and 10 mg/kg i.p. fluvoxamine groups. In the test phase, 10 mg/kg i.p. fluvoxamine reduced the CR (Fig. 5B). For the CR, there were also significant differences across the groups (four-group ANOVA, $F(3, 28) = 12.8, p < 0.005$), with *post hoc* testing revealing significant differences between the control and experimental groups, and between the formalin and 10 mg/kg i.p. fluvoxamine groups.

2.2. Experiment 2: The selection of preferred or repellent odor

Of the six types of odors tested, a honey odor (mixture of bee honey and mango) was the most preferred, but repellent odor was avoiding (Fig. 6). There were significant differences across the groups (seven-group ANOVA, $F(6,28) = 7.13, p < 0.005$), with *post hoc* revealing significant differences between the saline and honey+mango groups, and between repellent and other groups.

2.2.1. The effect of preferred (honey+mango) odor on the UCR and CR

Exposure to the honey+mango odor on day 1 of the training phase significantly reduced the mean time spent licking the hind-paw (Fig. 7A). For the UCR, there were significant

differences across the groups (four-group ANOVA, $F(3,28) = 108.9$, $p < 0.001$), with *post hoc* testing revealing significant differences between the control and experiment groups, and between formalin control and formalin honey+mango odor groups.

Exposure to the honey+mango odor in the test phase also significantly reduced the mean time spent licking the hind-paw (Fig. 7B). Again, there were significant differences across the groups (four-group ANOVA, $F(3,28) = 20.5$, $p < 0.005$), with *post hoc* testing revealing significant differences between the control group and experiment groups, and between the formalin control and formalin honey odor groups.

2.2.2. The effect of repellent odor on the UCR and CR

Exposure to the repellent odor on day 1 of the training phase significantly decreased the mean time spent licking the hind-paw (Fig. 8A). For the UCR, there were significant differences across the groups (four-group ANOVA, $F(3,28) = 31.9$; $p < 0.001$), with *post hoc* testing revealing significant differences between the control and experimental groups, and between the formalin control and formalin repellent odor groups. Repellent odor given in the test phase did not affect the CR (Fig. 8B).

3. Discussion

There have been very few studies comparing the psychopharmacological effects of analgesic drugs and olfactory stimulants on the CR, the conditioned pain response or a learned pain behavior. Our findings show that the analgesic drugs acetaminophen, fentanyl, gabapentin and fluvoxamine inhibit the UCR in a dose-dependent manner, but did not influence the CR once it was established. This fact proved the certainties that the CR have similar characteristics with chronic pain. Sweet-odor, olfactory stimulants, however, inhibited both the UCR and CR which had been developed. These results suggest that, for the CR, analgesic drugs and sweet odors affect different neural pathways and involve distinct mechanisms.

3.1. The influence of analgesic drugs on the UCR and CR

Acetaminophen reduced the UCR but did not affect the CR. Acetaminophen has been used for the treatment of acute and chronic pain, and is particularly effective against pain associated with edema. Acetaminophen has central antinociceptive actions at the level of the spinal cord and is very widely used in the management of acute and chronic pain (Bonnefont et al., 2003). Fentanyl reduced the UCR but did not affect the CR. Fentanyl acts on the dorsospinal nuclei and descending inhibitory pain pathways in the same way as morphine and other μ -opioid receptor agonists. At the supraspinal level, fentanyl acts in the periaqueductal gray matter area of the midbrain that receives projections from the amygdala, brainstem reticular formation, hypothalamus and the raphe nuclei (Miranda et al., 2012). Gabapentin acts throughout the limbic structures including the hippocampus and the sensorimotor system. In this study, gabapentin reduced the UCR but did not affect the CR. Gabapentin is an anti-epileptic agent but is also recommended as a first-line agent for neuropathic pain. It acts on the presynaptic

GABAergic terminals in the locus coeruleus and presynaptically reduces GABAergic synaptic transmission. Consequently, in neuropathic pain states, the inhibitory influence on neurons in the locus coeruleus is removed, leading to activation of the descending noradrenergic system (Kukkar et al., 2013; Snedecor et al., 2013). Our finding that the CR was resistant to gabapentin may explain the limitations of this drug in the treatment of chronic pain syndromes that include a CR. Certain antidepressants have become a part of the therapeutic strategy for different types of persistent pain including neuropathic pain, fibromyalgia, lower back pain and headache, and they are also used to treat the pain of osteoarthritis; SSRIs have a low or inconsistent analgesic efficacy, but better overall safety and tolerability compared with tricyclic antidepressants (Grégoire and Neugebauer, 2013). The mechanisms of action of antinociceptive antidepressants are complex, and although they act at a supraspinal, spinal and peripheral level, their effects are primarily thought to be due to their action at the descending inhibitory systems when these drugs are administered systemically (Mico et al., 2006). We found that fluvoxamine inhibited the UCR in a dose-dependent manner; however, it affected the CR at a larger dose. The fact that the CR was resistant to anti-inflammatory, opioid, anticonvulsant and a middle dose of fluvoxamine supports previous indications that the CR will have characteristics similar to intractable chronic or subacute pain in animals and humans (Nakama-Kitamura and Kitamura, 2011). The signs and symptoms of neuropathic pain are reported to disappear about 1 hour after formalin injection, but the injury to nerve endings causes functional and biochemical changes in other parts of the affected peripheral nerve and eventually to higher order neurons in the spinal cord and brain, such as anterior cingulate cortex (Kim et al., 2010). Nevertheless, the CR appears only in the conditioned context and is diminished to baseline by an extinction

procedure (Nakama-Kitamura and Kitamura, 2011). This also confirm that a sustained or chronic pain must involve a conditioning response, the CR. These observation can be attributed to a difference in the neural pathways of the CR and the action of analgesic drugs: the CR will mainly dependent on memory at a supraspinal level, but analgesic drugs stimulate the descending inhibitory neuronal systems or inhibit ascending system of pain including the dorsospinal nuclei as described above.

3.2. The effects of olfactory stimulants on the UCR and CR

Pain pathway in rodents and humans are similar in many ways (Sikandar et al., 2013). The mechanisms underlying the pain modulating effects of odors in human are not understood, but it has been reported that sweet smells can induce a physiological increase in pain tolerance (Prescott and Wilkie, 2007). The acute analgesic effect of lemon odor in mice is reportedly mediated by the descending inhibitory system controlled by the anterior cingulate cortex, an area known to mediate the emotional component of pain (Ikeda et al., 2014). We found that the attracted odors in rodents were different from those considered pleasant by humans: mice had no preference for complex or emotional odors such as jasmine, peppermint, lavender, lemon or vanilla but attracted sweet odors with strong, heavy fragrances such as honey, mango and ukon. Honey is a rich energy source and ukon has a savory smell and an antioxidant action with polyphenol in its ingredients: emotional in human vs. instinctive in animal.

Mutations reducing the function of Nav 1.7 sodium channels entail diminishing pain perception and olfactory acuity, suggesting a links between nociceptive and olfaction at ion channel level (Heimann et al., 2013). In this study, preferred odor inhibited the UCR, impaired the development process of the CR (data not shown), and developed the CR. These facts are clear contrast with analgesic drugs that did not affect the CR. A small

dose of the amnesic drug scopolamine, which had no effect on the UCR, inhibited the development process from the UCR to the CR. Mice exposed to preferred odor that had been pretreated with scopolamine did not develop a UCR or CR (data not shown), suggesting that attractant odors directly inhibit the CR irrespective of memory. The supraspinal experience of pain is strongly influenced by stress and attention, but reportedly alter the sensory and affective dimension of pain in different ways by different neural pathways (Villemure and Schweinhardt, 2010).

3.3. The difference in the potential underlying mechanisms of analgesic drugs and olfactory stimuli on the UCR and CR

Recently, it has been shown that congenital insensitivity to pain is accompanied by anosmia in mice and humans (Ahn et al., 2011; Weiss et al., 2011) and that voltage-dependent sodium channels Na(v)1.7 in olfactory sensory neurons are linked to learned responses, including aversive responses (Kobayakawa et al., 2012). Their results do not contradict our finding that preferred odors suppressed the nociceptive CR. Repellent odor, however, inhibited the UCR alone and after pretreatment with scopolamine, repellent odors minimally interfered with the inhibition of the development of the CR (data not shown), suggesting repellent odors may not affect the consolidation or memory retrieval mechanisms of pain. The decrease in nociceptive response might, in part, be related with a mitigation of attention to pain (Villemure et al., 2003). The effect of repellent odor on the CR requires further investigation.

In conclusion, analgesics such as acetaminophen, fentanyl, gabapentin and fluvoxamine exert their therapeutic actions mainly by activating the suppressing descending pain pathways at the spinal level. Therefore, pain perception may persist as a consequence of learned perception of pain, even after treatment with analgesics. The

sites of action of these drugs may explain why chronic pain is frequently unresponsive or incompletely responsive to analgesics. Conversely, preferred odors can suppress the activity of the neural pathways involved in the cognitive memory of contexts and, secondary innervated descending pain inhibitory system, and hence the CR to pain. Our findings suggest that targeting the olfactory system could be a novel means of treating intractable pain.

4. Experimental procedures

4.1: Experiment 1: The effect of analgesic drugs on the UCR and CR

4.1.1. Animals

The animals were experimentally naïve, adult, male specific pathogen-free (SPF) Jbc:ddY mice aged between 34 and 36 days and weighing 27.5–34.2 g at the beginning of the experiment. Animals were obtained from a colony of SPF mice maintained by Kears Co. Ltd., Osaka, Japan. The mice were housed four per cage in plastic cages (40 × 35 × 25 cm) and had free access to water and food (Oriental MF Yeast Co. Ltd., Osaka, Japan). The cages were kept at 21±1 °C and 55–60% humidity with a 12-h light-dark cycle (lights on from 07:00 to 19:00). Animals were acclimatized to the home cages for at least 4 days before the experiments. All experimental protocols were approved by the animal care committee of the Psychology Department of Kwansei Gakuin University, and undertaken in accordance with the National Institute of Health's guide, "Principles of Laboratory Animal Care" (NIH publication No. 85–23, revised in 1985), and the Kwansei Gakuin University Regulations for Animal Experimentation "Guide for the Care and Use of Laboratory Animals" (1992; revised in 2007).

4.1.2. Contexts

Two different contexts that differed in form, material, lighting conditions and perceptual stimuli were used. One context was a transparent polypropylene box ($13.5 \times 9 \times 5.5$ cm) with a mesh floor that was placed in an experimental room illuminated by a fluorescent lamp (40 W) 1.5 m above the boxes. The other context was a black polypropylene cylindrical box with a small window (diameter, $10 \times$ height, 7 cm) that was placed on a table with a transparent top illuminated by a 3 V light placed 50 cm away to allow observation of behavior from below. The experimental room was maintained at 22 °C (± 0.5 °C).

4.1.3. Drugs

Formaldehyde solution (37%; Wako Co. Ltd., Osaka, Japan) was diluted with sodium chloride solution (0.9% w/v) to produce 0.25% formalin. Acetaminophen (powder; Yamanouchi Co. Ltd., Tokyo, Japan) was dissolved in physiological saline solution at 70 °C to make 100, 150, and 200 mg/kg solutions and was injected i.p. at a volume of 0.1 mL/10 g. Fentanyl citrate for injection (0.1 mg/2 mL ampule, Sankyo Co. Ltd., Tokyo, Japan) was dissolved in saline to make 0.01 and 0.025 mg/kg solutions and was injected at 0.01 mL/10 g i.p. Gabapentin (powder; Cayman Chemical Co., Ann Arbor, MI, USA) was dissolved in saline (at 0.5 °C) to make 5 and 10 mg/kg solutions and was injected at 0.1 mL/10 g i.p. Fluvoxamine maleate (powder; Tocris Cookson, Ellisville, MO, USA and Bioscience Co., CA, USA) was dissolved in saline (at 0.5 °C) to make 5 and 10 mg/kg solutions and was injected at 0.1 mL/10 g i.p. Control mice were injected with an equal volume of saline.

4.1.4. Basic protocol

Experiments were performed between 10:00 am and 3:00 pm to avoid the effects of circadian factors, and every mouse in each group was tested at the same time on each

day. The context boxes were cleaned after each use with a 77% alcohol solution and washed using saline. The timeline of the experimental procedures were shown in Fig. 1.

4.1.4.1. Acclimatization phase

The acclimatization phase began 24 h to 48 h before day 1. During the acclimatization phase, four mice at a time were transported in an opaque plastic box (15 × 20 × 15 cm) to the experimental room from the home cage and allocated one each to four context boxes (two transparent or two black boxes) according to the experimental design. After 30 min, each mouse was taken from the box and returned to the home cage. This was repeated on the second day, with each mouse being placed in the alternate context box. Thus, mice were exposed to both the transparent and black boxes prior to the training phase.

4.1.4.2. Training phase

The UCR was measured on day1, and the CR was established on days 1 and 2 of the training phase, and measured in the subsequent test phase. On day 1, mice in the UCR groups were intraperitoneally injected with drug or saline in the home cages 10 min before transport to the experimental room and allocated to a context box. After 5 min in the context box, the formalin test was carried out (Rosland, 1990). Mice were exposed to an auditory stimulus (compound middle-pitch tone, 2800–3200 Hz, 75 dB, 5 s, 1 s/1 s, on/off; National B14-210) immediately before formalin injection. Using a 26-gauge needle connected to a microsyringe for tuberculin injection, 20 µL of 0.25% formalin was injected subcutaneously (s.c.) into the plantar surface of the left paw. The injection was undertaken near the floor of the box. Licking (including biting) behavior toward the injected hind-paw was used as a measure of the nociceptive response. Animals that licked (or bit) for 4 s or more were considered to have shown a positive response,

whereas those who responded for 3 s or less were considered to have shown no response, as short duration complex behaviors such as licking are a feature of normal mouse behavior. The mean duration of response was calculated per 5-min period for a total observation time of 30 min. The responses were closely observed by a skilled experimenter trained in the preparatory experiments and who was blind to group assignment. For the CR measurement, formalin was given in the left and right hind-paw on days 1 and 2, respectively, because repeated injection of formalin in the same paw diminished the nociceptive response. Parts of the behavior were recorded using a digital movie camera (Nikon, Coolpix S50, high sensitivity 1600, liquid crystal monitor) for confirmation.

4.1.4.3. Test phase

On day 4 of the experiment, mice in the CR groups were injected i.p. with saline in their home cages. After 10 min, they were transported to the experimental room and placed in the appropriate box according to the experimental design. After 5 min, mice were exposed to the auditory stimuli and 20 μ L of saline was injected s.c. into the plantar surface of the left hind-paw. Each mouse was immediately returned to the context box for observation. The time spent licking (including biting) was recorded for 30 min.

4.2. Experiment 2: Selection of preferred or repellent odor

4.2.1. Animals

Animals were treated according to the protocol outlined in Experiment 4.1.1 except that the animals were aged between 30 and 32 days and weighed 27.0–28.5 g at the beginning of the experiment.

4.2.2. Measurement of preferred or repellent odor

Innate olfactory preference or aversive reaction was evaluated using a preference

apparatus with three compartments (Jahangeer et al., 1997). Both sides of the central compartment (A) had a movable side wall and the opposite walls of the left (B) and right (C) compartments comprised a small container to accommodate a gauze swab, soaked with either olfactory stimulant substances or saline. The mouse was placed in the center compartment (A) and the slides opened for 15 min to allow it to become accustomed to the compartment. The times spent in compartments (B) and (C) were measured for 15 min using two stopwatches. The lid of compartment (A) was opened during measurements to minimize diffusion of odor between (B) and (C). To minimize the effect of any visual and spatial cues, the next day the apparatus was rotated by 180°, and the mean values of the two measurements were used for calculation: $[(B) / (B+C) \times 100]$.

4.3. Experiment 3. The effects of olfactory stimuli on the UCR and CR

4.3.1. Animals

Animals were treated according to the protocol outlined in Experiment 4.1.1 except that these animals were aged between 32 and 34 days and weighed 28.5–29.5 g at the beginning of the experiment.

4.3.2. Contexts

The contexts were the same as those used in Experiment 4.1.2.

4.3.3. Olfactory stimulant substances

The odor substances were honey (Kyoto, Japan), flute butter tropical mango (Sunpalko Co. Ltd., Kyoto, Japan), ukon (turmeric; Bio Actives Japan Co. Ltd., Tokyo, Japan) and aromatic flavors such as lavender, rosemary, roman chamomile, and eucalyptus. The repellent odor was particular to mice (mouse cube[®]; Sankyo Shodoku Shoji Co. Ltd., Tokyo, Japan).

4.3.4. Basic protocol

Experiments were performed in the same way as described Experiments 4.1.4. A timeline of the experimental procedures is shown in Fig. 1.

4.3 4.1. Acclimatization phase

The animals were treated according to the management outlined in 4.1.4.1, but a mesh sheet was placed on the floor of each box to accommodate filter paper soaked with saline.

4.3.4.2. Training phase

The UCR was measured on day 1 of the training phase. The animals were treated according to the protocol outlined in 4.1.4.2. except for no injection of drugs or saline in the home cage. A mesh sheet was placed on the floor of each box to accommodate filter paper soaked with odor or saline.

4.3.4.3. Test phase

On day 4 of the experiment, mice in the CR groups were treated in the same way as those in the Experiment 3.1.4.3. except that saline was not injected in the home cages. A mesh sheet was placed on the floor of each box to accommodate filter paper soaked with odor or saline.

4.4. Statistical analysis

For all experiments, analysis of variance (ANOVA) was applied to the data. When ANOVA revealed differences among the groups, pairwise comparisons of each experimental group versus the control group were performed using Ryan's test. The level of significance was set to $p < 0.05$.

Acknowledgments

We are grateful to Dr. Masahiko Shibata for his valuable guidance on variable patients with chronic pain. We thank Dr. Sadahiko Nakajima for his encouragement, Dr. Maya Suzuki for her advice on data analysis and help with the experiments, and Dr. Nobutaka Doe for his proofreading of the manuscript, and Seiji Tamakoshi for his support with fast Fourier transform analysis.

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Captions

Fig. 1. A time line of the experimental procedures.

Fig. 2. Effect of acetaminophen (ac) given in the training or test phase on the unconditioned (UCR) and conditioned nociceptive responses (CR). The mean duration of paw licking (including biting) in 5-min periods over 30 min is shown. A: On day 1 of the training phase, saline (s) or ac (100 or 150 mg/kg intraperitoneally; i.p.) was administered to mice in their home cage. After 15 min, saline (S) or 0.25% formalin (F) was given subcutaneously (s.c.) into the plantar surface of the hind-paw of mice that had been transferred to the experimental box, and the UCR was measured. B: In the test phase, s or ac was given in the home cage. After 15 min, S was injected in the experimental box and the CR was measured. Tone was given immediately before the injection of S or F. Error bars represent the mean $\pm$ standard error ($n = 8$). $*p < 0.05$ when comparing control mice with those that had received acetaminophen.

Fig. 3. Effect of fentanyl (fn) given in the training or test phase on the unconditioned (UCR) and conditioned nociceptive responses (CR). The mean duration of paw licking (including biting) in 5-min periods over 30 min is shown. A: On day 1 of the training phase, saline (s) or fn (0.01 or 0.025 mg/kg intraperitoneally; i.p.) was administered to mice in their home cage. After 15 min, saline (S) or 0.25% formalin (F) was injected subcutaneously (s.c.) into the plantar surface of the hind-paw of mice that had been transferred to the experimental box, and the UCR was measured. B: In the test phase, s or fn was given in the home cage. After 15 min, S was injected in the experimental box and the CR was measured. Tone was given immediately before the injection of S or F.

Error bars represent the mean $\pm$ standard error ($n = 8$). $*p < 0.05$ when comparing control mice with those that had received fentanyl.

Fig. 4. Effect of gabapentin (ga) given in the training or test phase on the unconditioned (UCR) and conditioned nociceptive responses (CR). The mean duration of paw licking (including biting) in 5-min periods over 30 min is shown. A: On day 1 of the training phase, saline (s) or ga (5 or 10 mg/kg intraperitoneally; i.p.) was administered to mice in their home cage. After 15 min, tone saline (S) or 0.25% formalin (F) was injected subcutaneously (s.c.) into the plantar surface of the hind-paw of mice that had been transferred to the experimental box, and the UCR was measured. B: In the test phase, s or ga was given in the home cage. After 15 min, S was injected in the experimental box and the CR was measured. Tone was given immediately before the injection of S or F. Error bars represent the mean $\pm$ standard error ($n = 8$). $*p < 0.05$ when comparing control mice with those that had received gabapentin.

Fig. 5. Effect of fluvoxamine (fl) given in the training or test phase on the unconditioned (UCR) and conditioned nociceptive response (CR). The mean duration of paw licking (including biting) in 5-min periods over 30 min is shown. A: On day 1 of the training phase, saline (s) or fl (5 or 10 mg/kg intraperitoneally; i.p.) was administered to mice in their home cage. After 15 min, saline (S) or 0.25% formalin (F) were injected subcutaneously (s.c.) into the plantar surface of the hind-paw of mice that had been transferred to the experimental box, and the UCR was measured. B: In the test phase, s, or fl was given in the home cage. After 15 min, S was injected in the experimental box and the CR was measured. Tone was given immediately before the

injection of S or F. Error bars represent the mean $\pm$ standard error ($n = 8$). $*p < 0.05$ when comparing saline control mice with those that had received fluvoxamine.

Fig. 6. Attractive (positive) or negative response to olfactory stimulation in mice. The response was evaluated by comparing the time (sec) spent in the compartment with the odor with a compartment that contained a saline control. Honey: bee honey ($n = 6$).

Fig. 7. Influence of preferred odor (honey/mango; h) given in the training or test phase of the unconditioned and conditioned nociceptive response (UCR and CR). The mean duration of paw licking (including biting) in 5-min periods over 30 min was shown. A: On day 1 of the training phase, filter paper soaked with saline (c, control) or h was placed under the mesh net on the floor of the experimental box, and saline (S) or 0.25% formalin (F) were injected subcutaneously (s.c.) into the plantar surface of the hind-paw of mice that had been transferred to the experimental box, and UCR was measured. B: In the test phase, filter paper soaked with c or h was put under the mesh net on the floor of the experimental box, S were injected into the plantar surface of the hind-paw of mice in the experimental box, and CR was measured. Tone was given immediately before the injection of S or F. Error bars represent the mean $\pm$ standard error ($n = 8$). $*p < 0.05$ when comparing control mice with those exposed to h.

Fig. 8. Effect of a repellent odor (r) given in the training or test phase of the unconditioned and conditioned nociceptive response (UCR and CR). The mean duration of paw licking (including biting) in 5-min periods over 30 min was shown. A: On day 1 of the training phase, filter paper soaked with saline (c, control) or r wrapped with filter

paper was placed under the mesh net on the floor of the experimental box, and saline (S) or 0.25% formalin (F) was injected subcutaneously (s.c.) into the plantar surface of the hind-paw of mice in the experimental box, and UCR was measured. B: In the test phase, c or r was placed in the experimental box, and S were injected into the plantar surface of the hind-paw of mice in the experimental box, and CR was measured. Tone was given immediately before the injection of S or F. Error bars represent the mean $\pm$ standard error (n = 8). * $p < 0.05$ when comparing control mice with those exposed to r.

Fig. 1. The timeline of the experimental procedures.

[Box] : Experimental box [Home cage] : Home cage
 T: tone F: formalin (s.c.) S: saline (s.c.) d: drug (i.p.) s: saline (i.p.) o: odor

Fig. 2. A., B

Fig.3. A. B.

Fig. 4. A.B.

Fig. 5. A. B.

Fig. 6.

Fig. 7. A. B.

Fig. 8. A. B.